PHYSIOLOGICAL AND ULTRASTRUCTURAL ASPECTS OF THE INFECTION OF CLOVER (*TRIFOLIUM FRAGIFERUM*) BY *RHIZOBIUM TRIFOLII* NA30

By

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To Anne-Marie, with much love and appreciation

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PHYSIOLOGICAL AND ULTRASTRUCTURAL ASPECTS OF THE INFECTION OF CLOVER (*TRIFOLIUM FRAGIFERUM*) BY *RHIZOBIUM TRIFOLII* NA30

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Chairman: Dr. David H. Hubbell
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Rhizobium trifolii NA30 was cultured in yeast extract-mannitol (YEM) broth, cell extract, and clover root exudate in order to study morphology and growth characteristics as related to the infection of clover.

In YEM broth *Rh. trifolii* NA30 had a generation time of 3 h. Aliquots of cells were taken from YEM broth to determine at what stage of growth the bacteria were most infective. Inoculum prepared from stationary phase cells at 72 h gave higher infection thread counts than inocula prepared from exponentially growing cells at 12 h or cells incubated in YEM broth for 39 days. To determine what effect the number of bacteria in the inoculum had on infection thread counts, 72 h cells were diluted in serial 10-fold dilutions and used as inocula. A

100-fold dilution in the number of bacteria in the inoculum resulted in a 22% reduction in the infection thread counts, while a 1000-fold dilution resulted in a 52% reduction. When examined graphically, the data conformed to an arithmetical increase in the infection threads per seedling with an exponential increase in inoculum size.

Rhizobium trifolii NA30 had a generation time of 3,5 h in soil extract. The cells entered stationary phase after 10 h of growth in this medium. Aliquots were taken at intervals from soil extract and inocula were prepared for infectivity studies. While growing exponentially in soil extract, the cells did not change their infectivity. Inocula harvested at 24 h and thereafter produced fewer infection threads per seedling.

Rhizobium trifolii NA30 had a generation time of 4 h in clover root exudate. The cells entered a stationary phase of growth after 12 h of growth in this medium. Aliquots were taken at intervals and prepared as inocula for infectivity studies. The mean number of infection threads per seedling increased as the cells aged in root exudate. After 7 days incubation in root exudate, *R. trifolii* NA30 produced 4 times as many infection threads per seedling than did cells incubated for 5 h in root exudate. The enhancement of infectivity induced by root exudate was more pronounced with stationary phase cells than with exponentially growing cells.

Electron microscopic examination of carbon-platinum shadowed *R. trifolii* NA30 cultured in YEM broth, soil extract, and clover root exudate revealed no complex life cycle. Cells cultured in each medium had a distinct rod morphology during exponential growth but tended to become more coccoid in the stationary phase. Binary fission was the

[illegible] good [illegible] cell division [illegible]. Asymmetrical cell divisions produced rods of various lengths. Cells cultured in soil extract and clover root exudate had asymmetrical divisions which resulted in the formation of cocci. The occurrence of cocci could not be correlated with infectivity. Cells cultured in root exudate became heavily encapsulated.

The light and electron microscopes were used to study the physical interactions between host and symbiont. The light microscope was used to study the initial response of clover root hair curling, to *R. trifolii* NA30. The intensity of root hair curling could be correlated with the number of rhizobia inoculated into the rhizosphere. With the light microscope it was possible to observe the rhizobia attached to the root hairs in a polar orientation. Infection threads could be observed at low magnification (150X). The newly initiated infection thread contracted only slightly with the cytoplasm of the root hair, but became progressively more light refractile with time. The root hair nucleus could be observed at the tip of the growing infection thread.

Ultrastructural studies of serial sections of infection threads in clover root hairs showed that the infection thread was initiated by an invagination process. Root hair wall growth was redirected at a localized point, resulting in the formation of an open pore. There was no direct penetration through the wall, and the bacteria remained extracellular within the root hair.

INTRODUCTION

The first stage in the establishment of the *Rhizobium*-legume N_2-fixing symbiosis is the infection of the host legume by the appropriate *Rhizobium* species. This is a highly specific interaction as each strain of *Rhizobium* is restricted to a particular group of legumes it can infect. These restrictions form the basis for speciation in *Rhizobium* and the so-called cross inoculation groups of host legumes and nodulating rhizobia.

In the clover symbiosis, infective strains of *Rhizobium trifolii* enter the host through root hairs. The bacteria enter the root hair and are enclosed in a tubular structure, the infection thread, which is the first microscopically visible sign of a successful infection.

The physiology of the rhizobia in the soil and the legume rhizosphere is an important consideration in understanding the infection process. The rhizobia are not obligate symbionts but can exist as free living heterotrophs and survive in soil for long periods of time away from the host plant. Little is known of the physiological condition of the cells during this period of existence in the soil in the absence of the host, and its possible relation to subsequent infection of an introduced host. As the rhizobia come under the influence of the host legume, the bacteria are stimulated to grow and divide. The physiology of the bacteria *in vivo* and *in vitro*, prior to and at infection, is unknown.

For this research *R. trifolii* NA30 was cultured in yeast extract-mannitol broth, soil extract, and clover root exudate to study the growth

of the bacteria as related to the infection of clover under simulated natural conditions. The morphology of the bacteria was followed to determine ultrastructurally whether the bacteria passed through distinct morphological changes which would constitute a life cycle as suggested by workers in the early 20th century

The light microscope has been invaluable in studying the growth of the infection thread through the root hairs However, the point of entry of the bacteria into the root, and thus the mechanism of infection, cannot be observed with the light microscope infections are initiated in tightly curled root hair tips, in areas where root hairs touch, or in areas covered by bacterial floes The electron microscopic examination of ultrathin serial sections was used as an approach in resolving this problem.

LITERATURE REVIEW

The rhizobia are aerobic, heterotrophic, Gram negative rods (0.5-0.9 µm by 1.2-3.0 µm) occurring singly or in pairs, generally motile when young by means of peritrichous, polar, or sub-polar flagella. The rhizobia can be arbitrarily classified as "fast growing" or "slow growing" strains. Fast growing strains produce acid and have a mean generation time of 2-4 hours. The slow growing strains produce alkali and have a mean generation time of 6-8 hours. Historically, the main taxonomic criterion for including a bacterium in the genus *Rhizobium* is its capacity to form morphologically defined nodules on the roots of a leguminous host. A symbiosis between *Rhizobium* and a non-legume, *Trema aspera*, which resulted in nodulation has been reported as an exception to the *Rhizobium*-legume specificity (42).

The rhizobia characteristically stain unevenly with the usual basic dyes. This uneven staining has been attributed to the accumulation of poly-β-hydroxybutyrate (34). Craig and Williamson (15) have identified polyphosphate, lipid, and glycogen in bacteroids of *Lotus*. Craig et al. (14) examined bacteroids of 83 strains of rhizobia and found five different inclusion bodies which included polyphosphate, poly-β-hydroxybutyrate, and lipid.

Early workers focused their attention on the pleomorphism of the rhizobia and postulated a complex life cycle as manifested by the existence of distinct morphological forms. Beijerinck (3) recognized three distinct forms of the rhizobia, swarmers or motile coccoid bodies,

typical rods, and bacteroids or coccium vacuolated forms. He reported the infective form of the bacterium to be the motile coccoid form. Schneidar (74) observed pure cultures of rhizobia in various stages of septation, budding, and branching. He suggested that division occurred as bi-septation, multi-septation, and budding and subsequent septation. Lohnis and Smith (49) recognized three distinct forms, straight rods, branched rods, and cocci. These forms were suggested to constitute a definite life cycle through which the organism normally passed.

Bewley and Hutchinson (4) found that the lack of available carbohydrate was conducive to a pre-swarmer form, while in the presence of available carbohydrate the cells would develop into a swarmer form and subsequently a rod form. They described a life cycle which they broke down into five stages.

1. *The swarmer form (non-motile, small coccus)*. When a culture was transferred to a neutral mail solution, it was converted after four or five days into the pre-swarmer form.

2. *Larger non-motile coccus*. When pre-swarmers were transferred to a medium containing carbohydrate, the original coccoid pre-swarmer increased in size until the diameter had doubled. At this stage the coccus remained non-motile.

3. *Swarmer stage (motile)*. The cell became ellipsoidal and developed high motility. This was recognized as the swarmer stage of Beijerinck (3).

4. *Rod form*. The swarmer proceeded to elongate and develop into a rod form which was still motile, but decreasingly so. As long as there was available carbohydrate, the organism remained in this form.

5 *Stage of high vacuolation* When the organism was placed back into a neutral soil extract (as the available carbohydrate was exhausted), it became highly vacuolated and the chromatin divided into a number of bands. Finally these bands became rounded off and escaped from the cod as the encoiled pre-swarmer.

Thornton and Gangulee (81) followed the changes in morphology of *Rhizobium* and found that a regular cycle of unbanded rods, cocci, and banded rods successively predominated in soil. The increase in the percentage of cocci was associated with increased bacterial numbers and with the appearance of motility. The time of appearance of the cocci could be controlled by modification of the culture medium. The addition of milk with 0.1% calcium phosphate would hasten the appearance of the coccus form and the movement of the bacteria through the soil.

Gangulee (26) repeated the experiments of Bowley and Hutchinson (4) and confirmed that phosphate hastened the appearance of the swarmer stage. The bacteria were examined in soil extract and all five stages of Bowley and Hutchinson's life cycle were found. Georgulee found that whether in liquid, agar, or soil, the various life cycle forms occurred simultaneously but in varying proportions. Soil conditions such as aeration, temperature, and presence of certain salts were among the factors that determined which stage predominated.

Various life cycles proposed the existence of a large "mother cell" inside which was contained a number of swarmer cells (for references, see 81). The swarmers were usually released in a non-motile condition, and afterwards developed flagella. The term gonidonglum was proposed by Lohnis (for reference, see 81) for this "mother cell" and the swarmers were called gonidia. Lewis (43) concluded that the gonidia were for-like inclusions

and have no relation to reproduction. He could find no complex life cycle associated with the rhizobia but did observe an orderly sequence in development as cells aged during early and late phases of growth.

Gibson (28) studied the morphology and reproduction of *Rhizobium*. He described such growth forms as rods, cocci, branched forms, gonidangia, gonidia, and mirrorcysts. Reproductive processes examined of fission, budding, liberation of gonidia, formation of regenerative bodies, and germination.

Gaw (27) studied the growth of the Vetch nodule bacteria in nitrate mannitol agar, Vetch extract, and soil extract and found that the bacteria did not pass through regular stages, although three principal cell types (cocci, unbranded rods, and branded rods) were observed.

Kinsel (5,6) examined a number of strains of *Rhizobium* from a wide variety of plants and observed distinct morphological forms which he regarded as a life cycle. Kinsel (6) also reported heat resistance in some species of *Rhizobium*. Kinsel and Hale (7) reported that tiny spherical swarmers were released from the cell lumen of sporulized large bacilli. Graham *et al* (30) were unable to demonstrate heat resistance when testing a large number of species of *Rhizobium*.

Dart and Mercer (18) obtained electron micrographs of *R. meliloti* on the surface of its host *Medicago* which showed structures they interpreted as "non-flagellated cells and multi-flagellated "swarmers". The swarmers ranged in size from 0.1 to 0.4 µm in diameter.

Dixon (21) and Dart (17) have reviewed the survival of rhizobia in soil. In summary, survival in soil is influenced by the plants which have grown there, the physical and chemical properties of the soil, and the strain of *Rhizobium*. The rhizobia can survive in soils in the

absence of legumes, although numbers are generally higher when the host legume is present. *Rhizobium meliloti* was reported to survive for long periods of time in sterilized soil amended with superphosphate and calcium carbonate with no loss of effectiveness (40). However, Nutman (59) found that *R. trifolii* frequently produced ineffective variants when grown in sterile soil.

Dudman and Brockwell (23) used gel immunodiffusion to study the persistence of *R. trifolii* introduced into soil by clover seed inoculation. They found that introduced populations of *R. trifolii* diminished with time and attributed the decrease to competition with naturally occurring rhizobia.

Fluorescent antibody techniques have been used as an approach to the study of *R. japonicum* in the soil (8,73); free living *R. japonicum* were detected in a variety of soils in the absence of the host legume.

There is a marked stimulation of *Rhizobium* numbers in rhizospheres, particularly of legumes, when compared with numbers found in soil more distant from the roots. Evidence for both stimulatory and inhibitory effects of legume root exudates on rhizobia has been reviewed by Dixon (21) and Dart (17). Legumes excrete a large number of substances into the rhizosphere, principally sugars, amino acids, and some vitamins (67,68). Legume root exudates stimulate *Rhizobium* growth (62) but not selectively enough in pure culture to account for differences in infectivity. Macgregor and Alexander (51) found that a non-invasive mutant of *R. trifolii* was a poor root colonizer. Many strains of *Rhizobium* need vitamins such as biotin and thiamin for growth, these are supplied in legume root exudates. Studies have indicated that nodulation specificity is not determined by selective stimulation (35)

or inhibitions (62) of rhizobia by legume root exudates. Different components of pea root exudates are stimulatory or inhibitory to the growth of *R. leguminosarum* (83).

Some legume seeds produce a water soluble, thermostable substance that is toxic in varying degrees to *Rhizobium* strains (12,80). The chemical nature of the toxin is unknown.

Root hairs are the site of infection by *Rhizobium* in a large number of legume species, particularly those of the families Trifolieae and Viciae. In the aquatic legume *Neptunia oleracea*, Schaede (72) did not find root hairs and proposed entry of the bacteria through the epidermal cells. Another important route of entry of rhizobia is the point of lateral root emergence (1,57).

The first microscopically visible indication of the bacteria-plant interaction is deformation and curling of the normally straight root hairs. A characteristic deformation is a curling of the root hair tip to produce a "shepherd's crook" (25). The bacteria enter the root hair and are enclosed in a tubular structure, the infection thread, which is the first visible sign of a successful infection (44). The majority of infected root hairs have the shepherd's crook at the infection thread origin, but exceptions exist (25,60). However, not all deformed root hairs contain infection threads.

Root hairs of non-legumes are not deformed by *Rhizobium* (31), and studies which tested several species of bacteria indicated that only *Rhizobium* caused deformation of legume root hairs (79). Root hair deformation is exhibited to some degree by both nodulating (homologous) and non-nodulating (heterologous) combinations of *Rhizobium* and legumes. However, a markedly curled condition of root hairs is almost always

restricted to the leguminous host associated with infective rhizobia (85) or their extracellular products (19)

The rhizobia produce several plant hormones including indole-3-acetic acid (13) and cytokinins (63). Indole-3-acetic acid (IAA) produced by the metabolism of tryptophan excreted by the legume root was thought initially to be responsible for root hair deformation. However, Nehlsen and Fahreus (69) demonstrated that IAA does not, at least alone, cause root hair curling. A strain specific extracellular rhizobial product has been demonstrated to induce root hair curling and deformation (19,34,47,76,85). Bubbeli (34) obtained deformation using a heat stable preparation obtained by alcohol precipitation. Ljunggren (47) found heat stable root hair deforming substances produced by rhizobia in the presence of the host. Solheim and Raa (76) have isolated several deforming substances which contained nucleic acid and protein or polysaccharide.

Rhizobium trifolii cells have polysaccharide antigens as their auriscea which are cross reactive with auriscae antigens on clover roots (15). Purified preparations of these polysaccharide antigens from infective strains of *R. trifolii* induced intense root hair deformation on *Trifolium fragiferum* while polysaccharide antigens from related noninfective mutants produced significantly less deformation when compared on a constant weight basis.

Single cells of *Rhizobium* have been observed attached to root hairs and epidermal cells in a polar (end-on) orientation (19,54,57,58,65,70). Polar attachment is not restricted to the *Rhizobium*-legume symbiosis and has been observed with bacteria attached to the epithelial surfaces in gastrointestinal tracts of mice (71). *Flavobacter* and *Hyphomorobium*

show polar orientation at solid-water and oil-water interfaces (53) in these cases polar orientation could not be explained by localization of surface ionogenic groups

The molecular basis for polar orientation of *Rhizobium* cells is unknown. Bohlool and Schmidt (9) suggested that specific interactions between rhizobia and the root hairs of the host legume may involve binding between legume lectins and the bacteria. Bohlool and Schmidt (10) have demonstrated that homologous fluorescent antibody to *R. japonicum* bound most heavily on one end of the cell. Fluorescein isothiocyanate labeled soybean lectin was also observed to bind predominantly at cell poles. The authors pointed out that it remained to be determined if polar lectin binding and polar antibody occur on the same end of the cell. Dazzo and Hubbell (19) have proposed a model wherein specificity in the *R. trifolii*-clover symbiosis is based on interactions of cross reactive surface antigens that are cross bridged by a multivalent clover lectin.

Several theories have been proposed regarding the entry of the bacteria into the root hair. Nutman (60) has advanced the hypothesis of root hair cell wall invagination. An invagination results from the reduction of plant cell wall growth at a localized point, resulting in the wall growing back into the root hair to form the tubular infection thread. There is no penetration through the wall at the point of entry, and the bacteria remain extracellular, i.e., there is no direct contact with the host cytoplasm.

Nutman's theory of invagination has been challenged on several points. First, how the cell wall invaginates against the high hydrostatic pressure of the root hair is unknown (21). Secondly, invagination

would form an open pore, which had not been shown in earlier electron micrographs (33,70) However, serial sections of root hairs were not used in these studies Additionally, an open pore would allow simultaneous entry of different cell types which would result in the presence of several *Rhizobium* strains in a single nodule. Early studies indicated that only one strain of *Rhizobium* was isolated from a nodule when the host had been inoculated with a mixture of infective rhizobia differentially marked by antibiotic resistance (51) or serological type (35,42) However, recent studies (41,46) have shown that several strains can be isolated from one nodule.

Ljunggren and Fahraeus (48) have proposed a "polygalacturonase" hypothesis in which the rhizobial exopolysaccharide increases plant pectic enzyme activity and a single bacterial cell softens and subsequently penetrates the plant cell wall without pronounced structural disruption The infection thread is presumably initiated once the bacterium penetrates to the plant plasmalemma

In support of this theory these workers demonstrated that a crude preparation of extracellular polysaccharide of infective rhizobia increased the activity or *de novo* synthesis of plant produced pectinolytic enzyme This activity was strain specific in that it correlated with the plant-bacterium specificity Munns (56) provided evidence to support this theory and demonstrated that the induction of pectinase (pectin transeliminase) was acid sensitive Bonish (11) found pectinolytic enzyme activity was not correlated with infectivity of strains In addition, other workers (45,52,75) have not been able to verify this hypothesis

The extracellular polysaccharide of *Rhizobium* has been well characterised in some cases. Hepper (32) found glucose, galactose,

glucuronic acid, pyruvate, and acetate in strains of *R. trifolii*. Small differences in composition between strains were not related to the ability to nodulate or to the capacity of the symbiotic organisms to fix nitrogen. Somme (77) examined extracellular polysaccharides from *R. meliloti*, *R. trifolii*, *R. phaseoli*, and *R. leguminosarum* and could find no significant differences in carbohydrate composition with the exception of *R. meliloti*, which lacked uronic acid. Methyl-O-glucuronic acid was a common constituent of *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* (29,36,37). Galactose has been identified in all the fast-growing strains as well as pyruvyl and acetyl substitutions (2,24,86). Cellulose has been identified as an extracellular product of some strains of *Rhizobium* (20,58).

Dudman (22) examined strains of *R. trifolii*, *R. leguminosarum*, *R. lupini*, and *R. phaseoli* and found both capsulated and bare cells, with the latter predominating. Dazzo and Hubbell (19) reported capsule formation in a strain of *R. trifolii*. This capsule was characterized as a high molecular weight ($> 4.6 \times 10^6$ daltons), β-linked, acidic heteropolysaccharide containing 2-deoxyglucose, galactose, glucose, and glucuronic acid.

Few workers have attempted ultrastructural studies of infected root hairs. Sahlman and Fahraeus (70) and Higashi (33) examined infected clover root hairs under the electron microscope. These authors did not section infected root hairs through the origin of the infection thread but did offer their micrographs in support of Nutman's theory of invagination (60). Dart (16) examined root hairs under the scanning electron microscope. He reported that root hairs and epidermal cells were coated with many bacteria, some of which appeared to be embedded in the wall. The root hair tips were often smooth but some older root hair surfaces had a fibrillar meshwork pattern.

MATERIALS AND METHODS

Bacterial strain--*Rhizobium trifolii* WA30, infective on *Trifolium fragiferum* was obtained from W. F. Dudman.

Media--Yeast extract-mannitol (YEM) broth (5B) was prepared, steamed for 15 min, filtered through Whatman No. 1 filter paper to remove excess $CaCO_3$, and sterilized by autoclaving.

Soil extract was prepared by steaming 1 kg air dried soil (2.96% moisture content) with 1 liter deionized water for 30 min. The extract was centrifuged at 13,200 x g for 10 min to remove soil particles, filtered successively through 5 µm and 0.45 µm membrane filters (Gelman Instrument Co., Ann Arbor, Mich.), lyophilized and stored desiccated. The dry weight of soil extract as prepared from 1 kg of soil in 1 liter of water was 440 µg/ml. Reconstituted soil extract was filter sterilized by passage through a 0.2 µm membrane filter.

Root exudate was prepared from *T. fragiferum* var. Palestine. The seeds were surface sterilized with 0.1% $HgCl_2$ for 10 min and rinsed extensively with sterile deionized water. The seeds were dispensed in petri dishes containing melted 2% water agar (Purified Agar, Difco Laboratories, Detroit, Mich.) tempered at 45 C, and the agar allowed to harden. The agar-seed slabs were transferred to sterile storage dishes (Corning No. 3250, 100 x 20 mm) which contained stainless steel mesh holders (16 mesh stainless steel wire cloth, Small Parts Inc, Miami, Fla.,) to position the slabs above but not touching the water. The seeds germinated through the stainless mesh into 50 ml of sterile

deionized water (pH 7.0). The root exudate was harvested after 7 days, filtered successively through 5 µm and 0.45 µm membrane filters, lyophilized, and stored desiccated. Clover root exudate as prepared had 190 µg dry weight/ml water. Reconstituted root exudate was sterilized by passage through 0.2 µm membrane filters.

Growth Conditions--Cultures were incubated on a rotary shaker (150 rpm) maintained at 25 C. Growth curves were determined by growing *R. trifolii* NA30 in appropriate media in nephelo culture flasks (Helios Giuss Inc., Vineland, N. J.) and measuring the optical density in a Bausch and Lomb Spectronic 20.

Harvesting--Rhizobium trifolii NA30 was grown in YEM broth and harvested at mid-exponential phase (12 h). Cells were centrifuged from YEM broth at 17,300 x g for 10 min in sterile Nalge 50 ml centrifuge tubes and washed twice in filter sterilized phosphate buffered saline (PBS; 0.05 M K_2HPO_4-KH_2PO_4, 0.15 M NaCl, pH 7.2).

Dry Weight Determinations--Cell mass determinations were made by filtering 25 ml samples through 0.4 µm Nuclepore filters (Nuclepore Corp., Pleasanton, Ca.). The filters were dried at 60 C for 20 min, cooled for 5 min, and weighed. This procedure was repeated at least twice to insure a stable weight. After sampling, the process was repeated to determine the weight of the bacteria.

Dry weight determinations of soil extract and root exudate were made by lyophilizing 5 ml of medium in acid cleaned, pre-weighed ampules. All samples were done in triplicate.

Bacteria-Plant Interactions--Trifolium fragiferum var. Palestine seeds were surface sterilized, rinsed and cold treated for 48 h at 4 C (61). Seeds were germinated overnight (inverted water agar plates)

into humid air at 22 C and transferred to Fahraeus glass-silde assemblies (25) inoculated with appropriate cultures. The assemblies were incubated in a plant growth chamber (Warren Sherer, Model CEL 255-6, Harahali, Mich.) programmed at 22 C isothermal, 12 h photoperiod, 18.6 lux light intensity.

Infectivity Studies—*Rhizobium trifolii* NA30, during growth in YEM broth (60), produced cellulose microfibrils which resulted in flocculation. Fibers were evenly dispersed and retained from 5 to 8 cells. Inocula were prepared for infectivity studies by centrifuging *R. trifolii* NA30 from media in sterile Nalge 50 ml centrifuge tubes at 17,300 x g for 10 min. Plate counts were determined by use of a Petroff-Hausser bacteria counter. Inocula were standardized with appropriate volumes of filter sterilized PBS. *Trifolium fragiferum* var. Palestine seedlings were inoculated and infection threads counted after 5 days incubation time using phase contrast microscopy.

Electron Microscope Studies—Equal volumes of 4% glutaraldehyde and bacteria in culture medium were mixed together. The bacteria were fixed for 1 h and washed successively with PBS and deionized water. Cells were dried on 200 mesh formvar coated grids and shadowed at approximately 45 degrees with carbon and platinum in a Balzers BA360M Freeze-etch apparatus (Balzers, Co., Furstentum, Leichtenstein).

For negative stained preparations, bacteria were centrifuged from YEM broth at 17,300 x g for 10 min and washed successively with PBS and deionized water. A drop of bacteria and a drop of 2% phosphotungstic acid (pH 7.0) were placed on a 200 mesh formvar-coated grid and allowed to set for 2 min. The excess liquid was drained from the grid and the grid was allowed to dry.

Three and 7 day old whole clover seedlings from Fahraeus assemblies

were fixed at 22 C for 2 5 h with 2 5% glutaraldehyde in 0 05 M cacodylate buffer (pH 6 8) and post-fixed at 22 C for 1 5 h with 1% buffered osmium tetroxide Seedlings were dehydrated through a graded ethanol series (25,50,75,95, and 100%) The 75% ethanol contained 2% uranyl acetate Acetone followed the ethanol series and the tissue was infiltrated with Spurr resin (7%) and polymerized overnight at 60 C The seedlings were first embedded in rectangular, Peel-a-way disposable embedding molds (22 x 40 mm; Peel-a-way Scientific, South El Monte, Ca-) The embedded seedlings were viewed with phase contrast microscopy, and areas containing infection threads were selected for sectioning Serial sections were cut on a Sorvall MT2 Ultramicrotome with a diamond knife The sections were picked up on formvar-coated one-hole grids and stained with Reynolds' lead citrate (66)

Bacteria were prepared for thin sectioning using the same procedure The cells were embedded in 1 5% agar after post-fixation with 1% osmium tetroxide. The agar was cut into small blocks (2-3 mm cubes) and the dehydration and infiltration with plastic continued

Carbon-platinum shadowed grids, negative stained bacteria, and grids containing thin sections were examined in a Hitachi HU11E electron microscope operating at 75 kV

RESULTS

Growth of Rhizobium trifolii NA30 in Yeast Extract-Mannitol Broth—Yeast extract-mannitol (YEM) broth is a standard laboratory medium used for the culture of *Rhizobium*. It was selected for use in this study because it is a complex growth medium in which nutrients would be in excess. Different morphological forms of *R. trifolii* NA30, if found during growth in this medium, would be characteristic of a true pleomorphic nature and not artifacts induced by nutrient limitation.

A growth curve (Fig. 1) was determined for *R. trifolii* NA30 so it would be known when the cells were actively growing (exponential growth) and in a stationary phase. *Rhizobium trifolii* NA30 had a mean generation time of 3 h in YEM broth. Fig. 1 shows that a 4% v/v inoculum had essentially no lag period when transferred to new medium. Cells were at mid-exponential phase in 10–12 h and in stationary phase at 24 h. As seen in Table 1 the pH of the medium became slightly acidic during the growth of the bacteria.

Rhizobium trifolii NA30 produced cellulose microfibrils (S#) and flocculated during all phases of growth in YEM broth. The flocs were evenly dispersed and contained from 5 to 8 cells. Aliquots of cells were taken from YEM broth at 12 h, 72 h, and 39 days and prepared as inocula for infectivity studies. Infection was defined as the ability to induce an infection thread in clover root hairs. Inocula for seedlings were standardized to 10^6 flocs/ml.

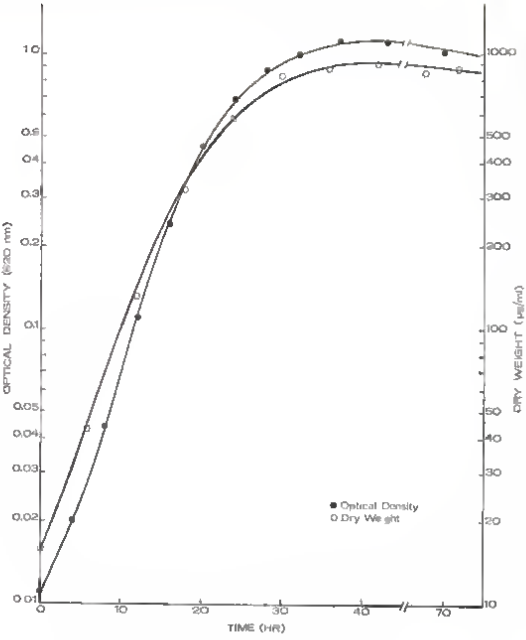


Fig 1 Growth curve of *Rhizobium trifolii* NA30 cultured in yeast extract-mannitol broth

Table 1 *Growth of Rhizobium trifolii HA30 in Yeast Extract-Mannitol Broth*

Time of Incubation (hr)	Optical Density 620 nm	Growth Phase	pH of Medium
0	011	Inoculation	7.0
6	030	Early Exponential	7.1
12	110	Mid-exponential	6.9
18	345	Late Exponential	6.9
24	700	Early Stationary	6.8
30	950	Stationary	6.7
50	950	Stationary	6.5

Table 2 gives infection thread counts after 5 days for the different ages of inocula. Inocula prepared from stationary phase cells at 72 h gave the highest infection thread counts (Table 2). As indicated by the high standard deviations for 72 h and 39 days, infection thread counts tended to be variable from one seedling to another for a given inoculum. This variability could perhaps be reduced somewhat by using an inbred variety of clover.

An analysis of the variance indicated that the mean number of infection thread counts for 72 h cells was significantly higher than for the other two cell ages at the 99% level of confidence. Inocula prepared from 72 h cells (stationary phase) was more infective than inocula prepared from 12 h or 39 day old cells. Infection threads were consistently initiated between 56 and 62 h after inoculation of the seedling. This initiation time remained constant and did not vary with the age of the inoculum. There is, then, at least a 56 h period of time when the bacteria may roam under the influence of the host. Cells at 72 h (stationary phase) were able to establish a more efficient interaction which led to infection.

Root hair adsorption was examined to determine if the age of the culture affected bacterial attachment. Bacterial suspensions consisting of single cells, as opposed to flocculated cells, were prepared by filtering cells through glass wool. Clover seedlings were set up in Fahraeus glass slide assemblies (25) and inoculated with single cell preparations from 12 h and 72 h *R. trifolii* NA30 cultured in YEM broth. There was no detectable difference in root hair adsorption between 12 h and 72 h cells (6 $\pm$ 2.7 and 6 $\pm$ 2.5 bacteria/root hair, respectively). The seedlings were examined after 12 h incubation in the dark. Bacterial attachment for 39 day old cells was not examined.

Table 2 *Influence of Inoculum Count from Trifolium pratense Inoculated Soil Rhizobium trifolii HA-0 Harvested from Yeast Extract-Mannitol Broth at Mid-exponential and Stationary Phase*

Trial No.	Age of Inoculum		
	12 hr	72 hr	39 days
1	33	145	30
2	18	85	40
3	29	112	34
4	24	92	61
5	25	122	26
6	24	98	44
Mean	26 ± 4.65	109 ± 25.3	39 ± 13.8

There were no detectable differences in root hair deformation on seedlings inoculated with 12 h and 72 h cells. Both preparations had markedly deformed root hairs which were shorter than uninoculated controls. These deformed root hairs ranged from 0.15 to 0.3 mm. The seedlings inoculated with 39 day old cells had longer root hairs than did uninoculated controls (greater than 0.6 mm).

To determine what effect the number of bacteria in the inoculum had on infection thread counts, 72 h cells were diluted in serial 10-fold dilutions and used as inocula. Table 3 gives the infection thread counts after 3 days. An analysis of the variance was performed on the infection thread counts given in Table 3. An F test indicated that, at the 95% level of confidence, there was a significant difference in the mean number of infection threads among the different sizes of inocula.

The mean number of infection threads was the same for the seedlings inoculated with 2.7×10^8 and 2.7×10^7 cfu/ml; however, the standard deviation for the latter was larger indicating a greater variance among counts. This inoculum (2.7×10^7) was able to induce 77 infection threads on one of the seedlings, which was a higher count than observed on any seedling inoculated with 2.7×10^8 cfu/ml. The inoculum prepared from 2.5×10^6 cfu/ml also gave a high count of 73 infection threads on one of the seedlings, but again there was a large variance in counts. This inoculum had a lower mean and a higher standard deviation than did the first two dilutions.

A 100-fold dilution in the number of bacteria in the inoculum resulted in a 22% reduction in the infection thread counts, while a 1000-fold dilution resulted in a 52% reduction. When examined graphically (Fig. 2), the data conformed to an arithmetical increase in the infection

Table 3 *The Effect of Varying the Inoculum Size on Infection Thread Counts*

Trial No	Flocs/ml			
	2.7×10^8	2.7×10^7	2.5×10^6	2.8×10^5
1	47	77	39	14
2	44	38	52	21
3	47	33	73	15
4	53	48	31	22
5	54	56	24	32
6	56	52	15	37
Mean	50 ± 4.4	51 ± 14	39 ± 19	24 ± 8.8

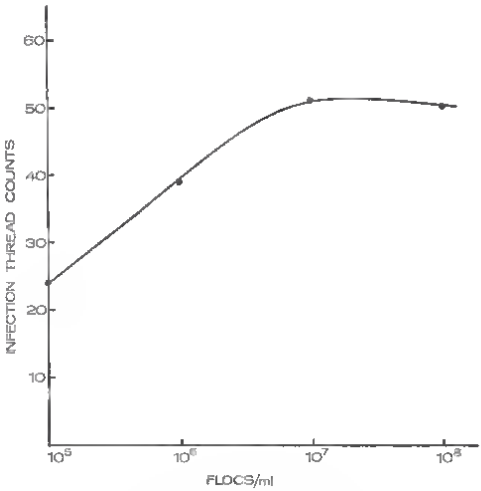


Fig. 2. Relation of infection thread counts to the number of bacteria in the inoculum.

thread per seedling with an exponential increase in the inoculum size, up to 10^7 flocs/ml, at which point a plateau was reached.

Morphology of Rhizobium trifolii NA30 in Yeast Extract-Mannitol Broth.--The morphology of *R. trifolii* NA30 was monitored by examining the cells under the light microscope using the standard Gram's stain and the electron microscope using carbon-platinum shadowed preparations.

Rhizobium trifolii NA30 had two types of inclusions, poly-β-hydroxybutyrate (PHB) and dark, polar inclusions which resembled polyphosphate bodies characterized in *Lotus* bacteroids by Craig and Williamson (15) and Craig *et al* (14). PHB was not apparent in carbon-platinum shadowed cells but could be seen in Gram stained preparations as non-staining areas. Polar bodies could be seen in carbon-platinum shadowed preparations when the bacteria were collapsed. If cells were not collapsed, they were too electron dense to discern polar bodies. Polar bodies could be seen occasionally in Gram stained preparations as nine polar inclusions within a Gram negative cell. The intensity of the blue color was variable among preparations which may have been a reflection of the extent of alcohol washing.

The representative morphology of the 48 h inoculum used for the growth curve in Fig. 1 is shown in Fig. 3 and 4. The predominant form of the bacteria at this time was a rod. Floc of cells were associated with cellulose microfibrils as indicated in Fig. 3. The bacterial cells were collapsed and the electron dense polar bodies could be seen (Fig. 3). The length of the rods was variable and this variability was attributed to slightly asymmetrical cell dividing as seen in Fig. 4.

Gram staining the culture at this time showed a homogeneous population of short, rounded rods. The cells were filled almost entirely with

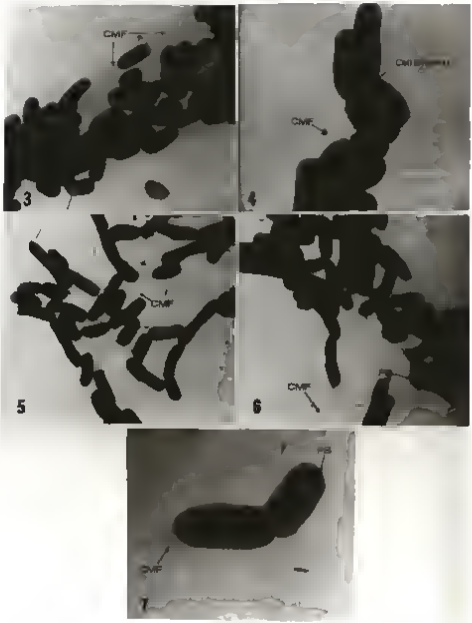
Fig 3. [illegible] shadowed 48 h [illegible] NA30 used as the inoculum for the growth curve. This micrograph shows a floc of rods with cellulose microfibrils (CMF). The rods are of various lengths. (X 6,068)

Fig 4. Carbon-platinum shadowed 48 h *N. trifolii* NA30 used as the inoculum for the growth curve. This micrograph shows a floc of rods which are of various lengths. The arrow labeled cell division indicates how an asymmetrical division has produced a short and a long rod. CMF indicates cellulose microfibrils. (X 11,556)

Fig. 5. Carbon-platinum shadowed 6 h *N. trifolii* NA30 showing a floc of rigid, electron dense rods. Cellulose microfibrils (CMF) are associated with the floc. The floc is comprised of rods of various lengths. (X 6,608)

Fig. 6. Carbon-platinum shadowed 12 h *N. trifolii* NA30 showing rods of uniform length. CMF indicates cellulose microfibrils and PB indicates polar bodies. (X 6,068)

Fig. 7. Carbon-platinum shadowed 18 h *N. trifolii* NA30 showing an asymmetrical division by which two rods of different lengths have been produced. (X 11,556)


CMF
3
CMF
4
CMF
5
CMF
6
CMF
7

PHB so that only the poles of the cells were stained. Some cells stained in a banded pattern which was attributed to the accumulation of PHB. Polar bodies were not seen in the Gram stained preparation.

Rhizobium trifolii NA30 was in early exponential growth 6 h after inoculation into YEM broth. The representative morphology of the cells at this time is shown in Fig. 5. The cells appeared more rigid at 6 h and were too electron dense to see polar bodies. The length of the rods was variable, and rods tended to remain attached and to form short chains and rosettes. Gram stained preparations at this time showed the cells were less vacuolated than the inoculum, but the cells continued to appear banded.

Fig. 6 shows representative morphology of *R. trifolii* NA30 at 12 h, which was mid-exponential growth. There was little difference in the morphology at this time when compared with cells at 6 h. The cells continued to appear rigid and cellulose microfibrils were associated with flocs of bacteria. At this time the length of the rods was the most uniform during growth in YEM broth. Gram staining of the cells at 12 h showed less vacuolation than at 6 h, but the staining of the cells appeared slightly banded.

At 18 h *R. trifolii* NA30 was in late exponential growth in YEM broth. Fig. 7 shows a shadowed preparation of cells at this stage. The cells were less electron dense, which indicated collapse. The cells were rigid during exponential growth but collapsed when in or approaching the stationary phase. When cells collapsed, it was possible to see the polar bodies. While the cells were still distinctly rod shaped at 18 h, a tendency toward shorter rods was apparent. Gram stained preparations of 18 h cells showed a slight increase in vacuolation.

The morphology of the cells of this strain was distinctly rod shaped when viewed under the light microscope.

After 18 h *R. trifolii* NA30 entered stationary phase. As seen in Fig. 8-11, as the cells aged in YEM broth, there was a tendency to become rounded and progressively more collapsed. Fig. 8 is a low magnification of a floc of 24 h rods shows cellulose microfibrils. A higher magnification of the cells showing more detail of the cellulose microfibrils and a polar body is shown in Fig. 9. In most cases, the cellulose microfibrils appeared to be restricted to the poles of the rods, but exceptions did exist. Fig. 10 and 11 show cells at 30 h and 48 h, respectively. As cells aged, the amount of cellulose microfibrils appeared to increase. By 48 h (Fig. 11) the cells were quite collapsed, even more so than the original 48 h inoculum (Fig. 3).

Gram stained preparations of stationary phase cells were considered in that the cells became highly vacuolated and staining was restricted to the poles of the cells. At 48 h, Gram stained preparations had distinct polar bodies within the cells. Fig. 12 shows a light micrograph of a Gram stained preparation of *R. trifolii* NA30. The arrows indicate the polar bodies which give the rods a banded appearance. The clear, unstained areas within the cells are attributed to the accumulation of PHB.

The banded appearance of the cells was also seen under the electron microscope with negative stained bacteria. Fig. 13 shows 48 h *R. trifolii* NA30 with a banded appearance due to the middle of the cytoplasm being electron transparent and the poles of the rods being electron dense. Within the electron transparent middle region were seen small, spherical, more electron transparent inclusions.

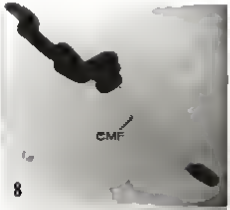
Fig. 8. Carbon-platinum shadowed 24 h *R. trifolii* NA30 showing a floc of collapsed rods which are different lengths. CMF indicates a long bundle of cellulose microfibrils. (X 5,612)

Fig. 9. Carbon-platinum shadowed 24 h *R. trifolii* NA30 showing a higher magnification of the cells seen in Fig. 8. The arrow indicates a bundle of cellulose microfibrils (CMF) at the pole of the rod. PB indicates a polar body in a short, rounded rod. (X 14,240)

Fig. 10. Carbon-platinum shadowed 30 h *R. trifolii* NA30 showing a floc of collapsed rods. (X 10,680)

Fig. 11. Carbon-platinum shadowed 48 h *R. trifolii* NA30 showing rods which are so collapsed that the morphology of the cells is difficult to discern. (X 10,680)

Fig. 12. A light micrograph of a Gram stained preparation of stationary phase *R. trifolii* NA30. The arrows indicate the darkly staining polar bodies which give the cells a banded appearance. (X 4,005)



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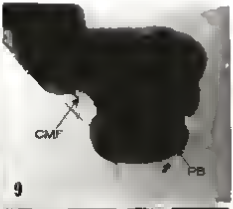

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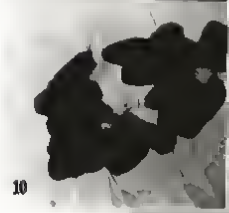

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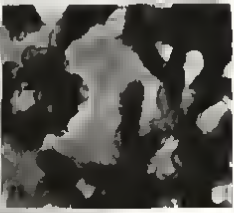

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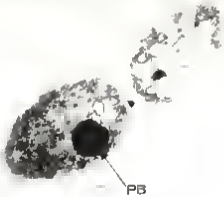
Fig. 13. Negative stained preparation of *R. [illegible]* NA30 showing bended cells (X 16,000)

Fig. 14 A thin section of *N. brufolii* NA30 showing electron transparent PHB inclusions (X 49,000)

Fig. 15. A thin section of *N. brufolii* NA30 showing an electron dense inclusion (X 63,000)

13

14



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In thin sections (Fig. 14 and 15) these inclusions were seen as small and diffuse or as larger electron transparent areas with no membrane surrounding them. The electron dense inclusions were also not surrounded by membrane, however, Fig. 15 shows membrane within the cytoplasm. Fig. 14 and 15 were 12 h, exponentially growing cells.

Harvesting Mid-exponential Cells--Rhizobium trifolii NA30 cells were harvested from YEM broth at 12 h for transfer to soil extract and clover root exudate. Cells at 12 h (mid-exponential growth) were chosen for transfer since, at this time, the cells had a uniform rod morphology (see Fig. 9 and 10). The purpose of these experiments was to show whether or not soil extract or root exudate would induce *R. trifolii* NA30 to undergo morphological changes which could be considered to constitute a life cycle.

Fig. 16 shows the effect of harvesting and resuspending cells in phosphate buffered saline (PBS, pH 7.2) or deionized water (pH 7.0). The optical density and dry weight of the cells was monitored for 9 h. The optical density of the water suspended cells decreased while the optical density of the PBS suspended cells remained fairly constant. Visual and microscopic examination revealed that flocculation was enhanced by suspending the cells in water. The decrease in optical density was attributed to flocculation. When PBS and water suspensions were examined on a dry weight basis, both decreased in mass at the same rate (Fig. 16). It was decided to use PBS to harvest and resuspend *R. trifolii* NA30 in order to avoid excessive flocculation.

Growth of Rhizobium trifolii NA30 in Soil Extract--The dry weight of soil extract as prepared from 1 kg of soil in 1 liter of water was 440 µg/ml. This concentration of soil extract was called 1X. Soil

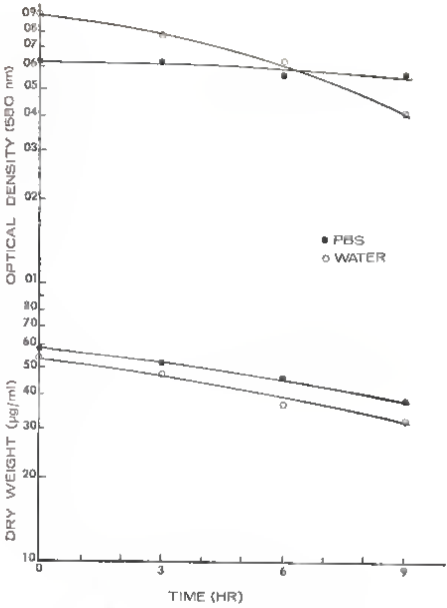


Fig. 16 Effect of suspending *Rhizobium trifolii* WA30 in phosphate buffered saline and water.

extract could not be concentrated greater than 4 times when using lyophilized soil extract. The dry material could not be completely reconstituted. The following concentrations of soil extract were prepared: 1 5X (660 µg/ml), 2.0X (880 µg/ml), and 4X (1 76 mg/ml). *R. trifolii* NA30 was harvested from YEM broth at 12 h (mid-exponential growth) and resuspended in the different concentrations of soil extract. The growth of the bacteria was monitored by optical density. As seen in Fig. 17, the total growth of the bacteria, rather than the generation time, was affected by the different concentrations of soil extract.

Rhizobium trifolii NA30 was harvested from YEM broth at 12 h and resuspended in 4X concentrated soil extract. The growth curve is shown in Fig. 18. The mean generation time of the bacteria in 4X soil extract was 3.5 h. By the time the cells entered stationary phase (10 h), the cell mass had approximately tripled. As indicated in Table 4, the pH of the medium increased during the growth of the bacteria in soil extract. The cells either produced alkaline end products or consumed an acidic substrate. There was no buffer added to the medium.

Aliquots of cells were taken from soil extract at intervals and prepared as inocula for infectivity studies. Total cell counts in the inocula were standardized to 10^6 flocs/ml. Table 5 gives infection thread counts after 5 days incubation. While the cells were growing exponentially (up to 10 h) in soil extract, the cells did not change their infectivity. Cells from stationary phase (at 24 h and thereafter) formed fewer infection threads per seedling. Due to the presence of nondispersible flocs, the viability of the culture could not be accurately determined. Infection threads were initiated within the time range of 56 to 62 h after inoculation. This time range did not vary with the age of the inoculum.

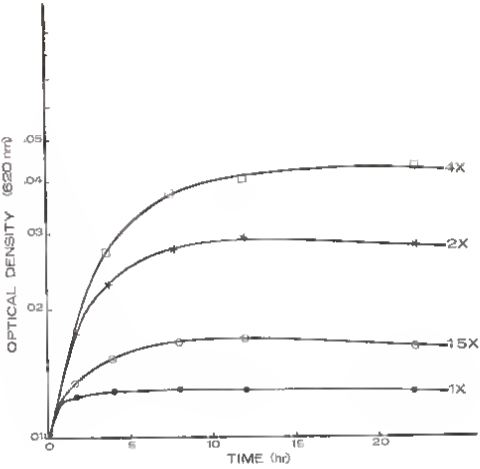


Fig. 17 Growth of *Rhizobium trifolii* NA30 in different concentrations of soil extract.

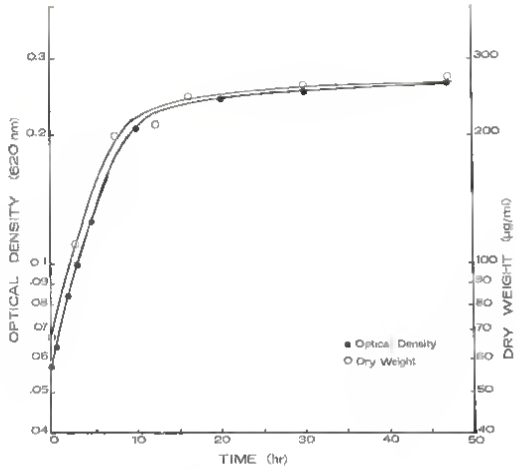


Fig 18 Growth curve of *Rhizobium trifolii* NA30 in 4X concentrated soil extract

Table 4. *Growth of Rhizobium trifolii NA30 Cultured in Soil Extract*

Time of Incubation (hr)	Optical Density 620 mm	Growth Phase	pH of Medium
0	.046	Inoculation	7.0
5	.108	Mid-exponential	7.3
10	.218	Early Stationary	7.4
24	.240	Stationary	8.0
48	.233	Stationary	8.0

Trial No	0 h	5 h	10 h	[illegible]	[illegible]	[illegible]
1	20	12	14	[illegible]	[illegible]	[illegible]
2	25	15	15	[illegible]	[illegible]	[illegible]
3	43	34	[illegible]	[illegible]	[illegible]	[illegible]
4	42	42	[illegible]	[illegible]	[illegible]	[illegible]
5	38	24	[illegible]	[illegible]	[illegible]	[illegible]
6	16	46	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Morphology of Rhizobium trifo[illegible]—Aliquots were taken at intervals to determine if *R. trifolii* NA30 passed through distinct morphological changes which could be called a life cycle as proposed by earlier workers (3,4,43,48,74).

Fig. 19 shows the representative morphology of *R. trifolii* NA30 harvested from YEM broth at 12 h. Cells at mid-exponential growth were fairly uniform in morphology. At that time most cell divisions were symmetrical and rods were of a uniform length. Cells at this stage of growth were too electron dense to see polar bodies.

The representative morphology of *R. trifolii* NA30 cultured in soil extract (4X concentrated) is shown in Fig. 20-25. Throughout growth in soil extract, the predominant morphological form of the bacteria was a rod. The rods remained rigid through 36 h (Fig. 10-23) but at 72 h (Fig. 24) the cells appeared collapsed.

Asymmetrical cell division resulting in the formation of coccoi became apparent at 6 h (Fig. 20). At this time approximately 5% of the cells had a mucus forming at the pole of the rod. The cocci were the same diameter as the rod. Formation of cocci continued through the 12 h sampling (Fig. 21) and 24 h sampling (Fig. 22). At 24 h the frequency of occurrence of cocci at the poles had decreased to 3% of the cells. It was not until 36 h (Fig. 23) that free cocci were detected in the medium. At this time free cocci in the medium constituted approximately 3% of the cells.

At the 72 h sampling (Fig. 24), the cells were collapsed. While morphology became somewhat obscured due to collapsing, the variability in rod length could be detected and cocci were seen at the poles of the rods. Before 72 h the cells were too electron dense to see polar bodies.

Fig. 19 Carbon-platinum shadowed *R. trifolii* NA30 harvested from YEM broth at 12 h. This micrograph shows the fairly regular morphology of the cells. (X 10,680)

Fig. 20 Carbon-platinum shadowed 6 h *R. trifolii* NA30 cultured in soil extract. The arrows indicate cocci which appeared to be forming at the poles of the rods. (X 5,607)

Fig. 21 Carbon-platinum shadowed 12 h *R. trifolii* NA30 cultured in soil extract. The arrows indicate cocci which appeared to be forming at the poles of the rods and bundles of cellulose microfibrils (CMF). (X 10,680)

Fig. 22. Carbon-platinum shadowed 24 h *R. trifolii* NA30 cultured in soil extract. The arrow points out a coccus. As seen in this micrograph the rods are of various lengths. Cellulose microfibrils are indicated by CMF. (X 4,094)

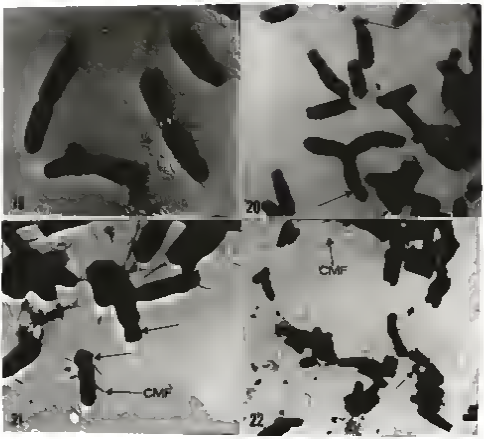
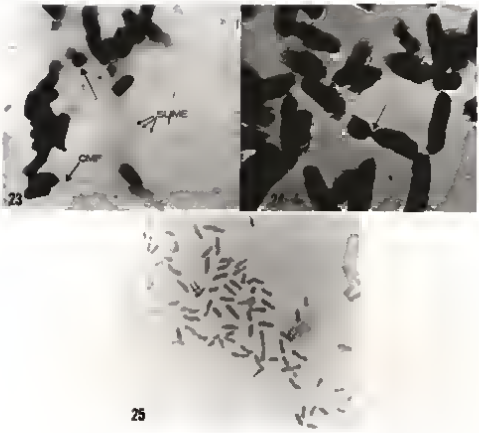

19
20
CMF
CMF
21
22

Fig. 23. Carbon-platinum shadowed 36 h *R. trifolii* NA30 cultured in soil extract. The arrow indicates a free coccus. The bacteria were surrounded by slime and cellulose microfibrils are indicated by CMF. (X 5,607)

Fig. 24. Carbon-platinum shadowed 72 h *R. trifolii* NA30 cultured in soil extract. The arrow indicates a rod and a coccus formed by asymmetrical division. (X 10,680)

Fig. 25. A light micrograph of a Gram stained preparation of *R. trifolii* NA30 at 12 h in soil extract. The arrows indicate the darkly staining polar bodies which give the cells a banded appearance. (X 4,005)


SLIME
CYST
23
24
25

Stationary phase cells remained rigid for a longer period of time when cultured in soil extract as compared with YEM broth. In YEM broth the cells remained firm during exponential growth, but as the bacteria entered the stationary phase, the cells were collapsed. In soil extract the cells remained firm during stationary phase (after 10 h) and up to 36 h. The explanation for this is unknown at this time.

Gram stained preparations of cells grown in soil extract showed banded rods throughout the incubation period. Fig. 25 shows a light micrograph of a Gram stained preparation at 12 h. The arrows indicate the darkly staining polar bodies which caused the cells to appear banded. After 12 h in soil extract the banding was so pronounced that the rods could have been misinterpreted as strings of cocci. Positive staining with crystal violet revealed that these were rods. For this reason it was difficult to resolve the actual cell. After 12 h there was little change in the Gram stained preparations. The rods had a tendency to become shorter and banding continued. After 36 h free cocci were not detected with the light microscope, as had been the case with the electron microscope. It was difficult to resolve the cocci from the banded rods. The difference in rod length was not resolved by the light microscope.

Cells were prepared for thin sectioning and freeze-fracturing. The low frequency of occurrence of cocci made it impossible to demonstrate budding at the poles of the rods.

Growth of Rhizobium trifolii NA30 in Clover Root Exudate--Clover root exudate as prepared had 190 µg dry weight/ml water. This concentration was called 1X. Root exudate could not be concentrated greater than 5X when using lyophilized root exudate since the dry material could not be completely dissolved.

Rhizobium trifolii NA30 was harvested from YEM broth at 12 h and transferred to root exudate prepared at the following concentrations: 1X (190 µg/ml), 2X (380 µg/ml), and 4X (760 µg/ml). Fig. 26 shows that the optical density of cells cultured in 1X and 2X root exudate decreased after the cells entered the stationary phase. The decrease in optical density was correlated with a decrease in cell mass, as seen in Fig. 27. Cells cultured in 4X concentrated root exudate showed a slight decrease in optical density and cell mass during a 36 h incubation period.

Rhizobium trifolii NA30 was harvested from YEM broth at 12 h and transferred to 5X concentrated root exudate. Fig. 28 shows the growth of *R. trifolii* NA30 in this medium. The optical density of the culture began to decrease after 22 h. The total cell mass also began to decrease at this time. The generation time of *R. trifolii* NA30 in 5X root exudate was 4 h. As indicated in Table 6, the pH of the root exudate increased slightly during growth of the bacteria.

The root exudate contained some seed coat exudate; it was thought that the seed coat exudate was causing the decrease in optical density and dry weight. There are reports of seed coat toxicity (12,30) in the literature. It has been observed during this research that when seed coats remained at the top of the cover slip used in the Fahraeus glass slide assemblies, a zone of inhibition of bacterial growth could be seen in the rhizosphere. The size of the zone of inhibition corresponded to the size of the seed coat.

The dried root exudate was thought to contain phenolic compounds because of the dark-brown color and the fact that phenolic compounds frequently occur in very high concentrations in plants. Loomis and Battaile (50) described a technique using insoluble polyvinylpyrrolidone

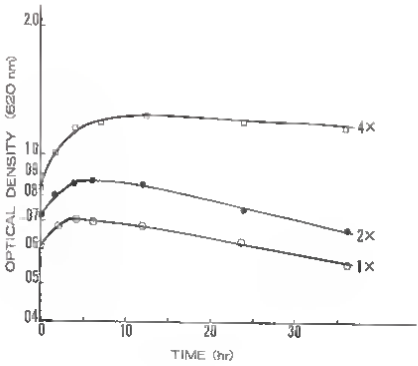


Fig 26 Growth response of *Rhizobium trifolii* NA30 to different concentrations of clover root exudate (optical density).

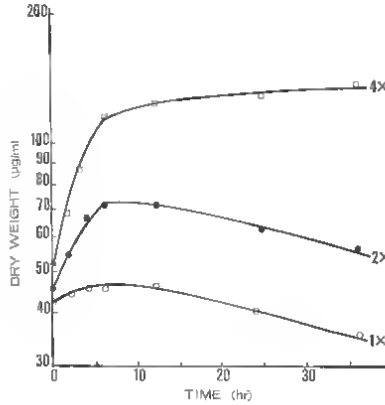


Fig. 27: Growth response of *Rhizobium trifolii* NA30 to different concentration of clover root exudate (dry weight)

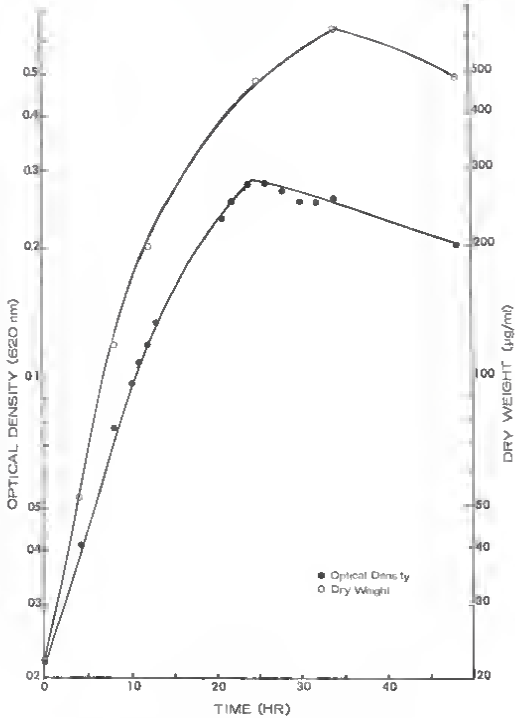


Fig 28 Growth curve of *Rhizobium trifolii* NA30 in 5X concentrated clover root exudate

Table 6. *Growth of Rhizobium trifolii HA30 Cultured in Clover Root Exudate*

Time of Incubation (hr)	Optical Density 620 nm	Growth Phase	pH of Medium
0	051	Inoculation	7.0
5	.105	Mid-exponential	6.9
10	.157	Late Exponential	7.0
24	177	Stationary	7.2
72	170	Stationary	7.3

(PVP) to adsorb phenolic compounds. The root exudate was filtered through glass columns (12 cm x 25 cm) packed with washed, insoluble PVP. This treatment removed the brown color of the root exudate.

Rhizobium trifolii NA30 was harvested from YEM broth at 12 h and resuspended in PVP treated root exudate prepared at the following concentrations: 1.7 mg/ml, 3.2 mg/ml, 4.0 mg/ml, 5.0 mg/ml, and 6.0 mg/ml. PVP treated root exudate could be reconcentrated more than untreated root exudate.

The growth curves of *R. trifolii* NA30 cultured in PVP treated root exudate is shown in Fig. 29. The mean generation time of cells cultured in PVP treated root exudate was 4.5 h. The generation time was the same for the different concentrations of root exudate, but the total growth of the bacteria was concentration dependent. There was no decrease in optical density with cells cultured in PVP treated root exudate.

Soybean seed extract has been used as a component in growth media in the place of yeast extract. A non-defatted soybean seed extract medium gave higher cell counts than did a yeast extract medium (38). Clover seed extract was prepared by the method of Dazzo and Hubbell (19). A medium was prepared which contained 20% clover seed extract (vol/vol) in root exudate (2.7 mg/ml). The addition of seed extract reduced the generation time of *R. trifolii* NA30 from 4.5 h to 2.5 h (Fig. 29). The effect of different concentrations of seed extract in the medium was not tested.

Inocula were prepared from cells incubated in PVP treated root exudate and standardized to 10^6 flecs/ml. Infection threads were counted after 5 days incubation. Infection threads were initiated in the time range of 56 to 62 h after inoculation when the inocula were prepared

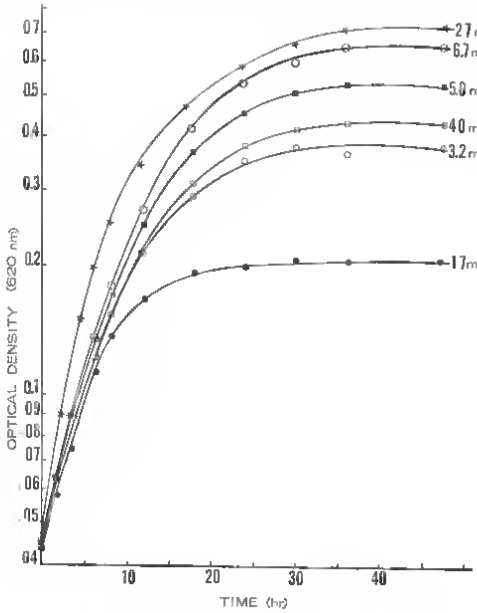


Fig. 29 Growth response of *Rhizobium trifolii* NA30 to different concentrations of FVP treated clover root exudate. The 2.7 mg treatment (asterisk) contained 20% clover seed extract.

from exponentially growing cells (through 10 h) Inoculo prepared from stationary phase cells at 24 h and thereafter initiated infection threads by 44 to 48 h.

The mean number of infection threads per seedling increased as the cells aged in root exudate (Table 7) After 7 days incubation in root exudate, *R. trifolii* NA30 produced 4 times as many infection threads per seedling as did cells incubated in root exudate for 5 h. The synchronism of infectivity induced by root exudate was more pronounced with inoculo prepared from stationary phase cells than from exponentially growing cells

Morphology of Rhizobium trifolii NA30 Cultured in Clover Root Exudate--Aliquots were taken from clover root exudate at intervals to determine if *R. trifolii* NA30 passed through distinct morphological changes which could be related to complex reproduction or constitute a life cycle

The representative morphology of *R. trifolii* NA30 cultured in root exudate is shown in Fig 30-42 The morphology of the 12 h inoculum harvested from YEM broth was identical to that seen in Fig. 19 The inoculo contained rods which were fairly uniform in morphology

After 4 h in root exudate there was an accumulation of both slime and capsular material which was not associated with the 12 h inoculum Slime was defined as the extracellular material which was loosely associated with cells A capsule was defined as that material which completely surrounded and had physical contact with the bacterial cells; Fig 30 shows cells which were embedded in a fibrillar matrix. The cellulose microfibrils radiated from the cells in bundles Fig 31 shows encapsulated cells. The capsular material next to the cells

[illegible] *from Root Exudate*

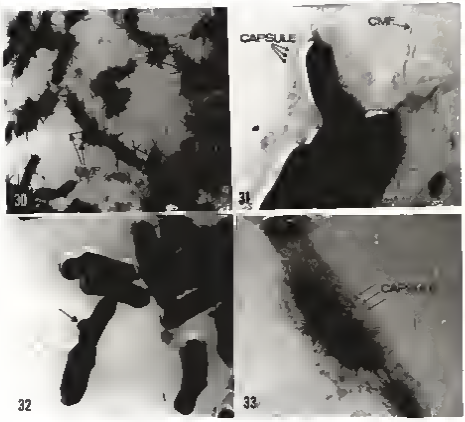
Trial No.	No. of Cells Harvested from Root Exudate					
	0 hr	5 hr	10 hr	24 hr	72 hr	[illegible]
1	25	39	46	65	98	12[illegible]
2	35	29	32	85	91	128
3	39	25	50	51	121	135
4	38	39	37	69	110	142
5	29	40	40	58	95	120
6	32	29	49	71	103	140
Mean	33 ± 12.1	33 ± 14.7	42 ± 18.4	67 ± 26.1	103 ± 27.6	136 ± 29.5

Fig. 30 Carbon-platinum shadowed 4 h *R. trifolii* NA30 cultured in root exudate. This micrograph shows rods which had cellulose microfibrils (CMF) arranged in bundles. A fibrillar material completely covers the background of the grid. (X 5,481)

Fig. 31 Carbon-platinum shadowed 4 h *R. trifolii* NA30 cultured in root exudate. A floc of cells is surrounded by a fibrillar capsule. (X 10,440)

Fig. 32 Carbon-platinum shadowed 8 h *R. trifolii* NA30 cultured in root exudate. The arrow indicates a bulge in the rod. (X 10,440)

Fig. 33. Carbon-platinum shadowed 8 h *R. trifolii* NA30 cultured in root exudate. The cells are completely surrounded by a fibrillar capsule. (X 7,830)


CAPSULE
CMF
30
31
32
33

appeared fibrillar but was smoother in appearance away from the cells. Cellulose microfibrils were seen associated with cells (Fig. 31)

Gram stained preparations showed cells more clumped at this time than in the 12 h inoculum from YEM broth. There was an increased amount of extracellular material around the cells.

At 8 h in root exudate it was difficult to distinguish cellulose microfibrils from flagella. After 4 h in root exudate there was a marked increase in the motility of the culture as seen by examining wet mounts under the phase microscope. Flagella may have been sheared from the cells and broken up due to the washing and centrifuging procedures used in preparing cells for shadowing. It is thought that the appendages seen in Fig. 32 were flagella. Not all cells were encapsulated (Fig. 32). However, as seen in Fig. 33, some cells were heavily encapsulated by a fibrillar material. At 8 h *A. tribolii* NA30 was becoming pleomorphic as seen by the budding cells in Fig. 32. At 8 h the cells were too electron dense to see the polar bodies.

Gram stained preparations at 8 h showed an increasing staining extracellular material around clumps of cells. While the morphology of the cells was a fairly uniform rod, there were a small number of swollen cells.

Fig. 34-36 show cells at late exponential growth (12 h). At this time polar bodies were seen free in the sediment. The cells were pleomorphic and the rods were of various lengths. Fig. 34 shows noncapsulated cells associated with released polar bodies. As seen in Fig. 35, slime was seen with some cells. The rods seen in this micrograph are of different lengths. Fig. 36 shows an encapsulated cell and a released polar body.

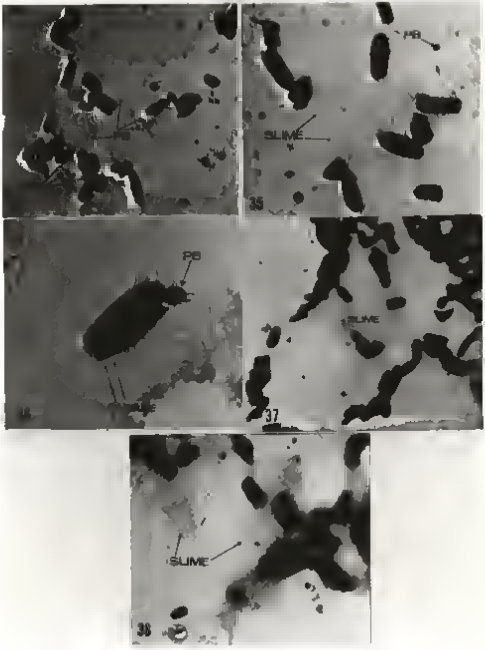
Fig. 34. Carbon-platinum shadowed 12 h *R. trifolii* NA30 cultured in root exudate. Rods of various lengths are seen. PB indicates polar bodies which have been released into the medium. (X 5,733)

Fig. 35. Carbon-platinum shadowed 12 h *R. trifolii* NA30 cultured in root exudate. Rods of various lengths are shown associated with slime. PB indicates a released polar body. (X 8,190)

Fig. 36. Carbon-platinum shadowed 12 h *R. trifolii* NA30 cultured in root exudate. An encapsulated cell is shown with a released polar body (PB). (X 18,200)

Fig. 37. Carbon-platinum shadowed 24 h *R. trifolii* NA30 cultured in root exudate showing cells of various lengths associated with slime. (X 4,186)

Fig. 38. Carbon-platinum shadowed 24 h *R. trifolii* NA30 cultured in root exudate showing rods of various lengths associated with slime. (X 5,733)


PB
SLIME
35
PB
SLIME
37
SLIME
38

Gram stained preparations at 12 h showed an increased amount of extracellular material. The cells could barely be seen, but appeared as banded rods when visible.

At 24 h (Fig. 37 and 38) the cells were still rigid and not collapsed. As seen in Fig. 37 and 38, the slime surrounded the cells. Short, rounded rods were seen at this time but no curri were observed. Gram stained preparations of 24 h cells appeared the same as the 12 h cells.

Cells at 48 h are seen in Fig. 39 and 40. At this time a number of curri were observed in the medium. Fig. 39 shows a raccus, and next to it a cell which appears to be branching. An aberrant cell is seen in Fig. 40. This appears to be a branched filament. A pleomorphic cell such as this was a rare occurrence. At 48 h the cells were less rigid and polar bodies could be seen in some cells (Fig. 40). Gram stained preparations at this time showed banded rods. The banding was so pronounced that a regular pattern of stained and unstained areas was seen. This banding made the cells (rods) appear as strings of curri. Positive staining with crystal violet revealed that these were rods.

At 72 h most cells were collapsed (Fig. 41 and 42). At this time polar bodies could be distinctly seen within the rods. The rods were of different lengths. Curri were not apparent at this time. As seen in Fig. 41, slime was associated with some cells. Gram stained preparations showed cells which were swollen and banded. The swollen cells were rounded rods.

Bacteria-Plant Interactions--Straight, undeformed root hairs, as seen in Fig. 43, occurred on axenically grown *Trifolium fragiferum*

Fig. 39. Carbon-platinum shadowed 48 h *R. trifolii* NA30 cultured in root exudate. The unlabeled arrow indicates a coccus. A pleomorphic rod is seen next to the coccus. (X 8,700)

Fig. 40. Carbon-platinum shadowed 48 h *R. trifolii* NA30 cultured in root exudate. A long branched filament is seen in the center of the micrograph. FB indicates the polar bodies in the bacteria. (X 8,700)

Fig. 41. Carbon-platinum shadowed 72 h *R. trifolii* NA30 cultured in root exudate showing a floc of collapsed rods which are various sizes. The cells are associated with slime and FB indicates the polar bodies. (X 8,700)

Fig. 42. Carbon-platinum shadowed 72 h *R. trifolii* NA30 cultured in root exudate showing a floc of rods which are various sizes. FB indicates polar bodies inside the cells and others which are released into the medium. (X 6,100)

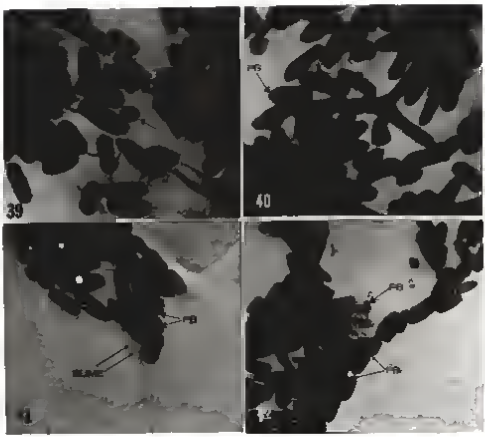
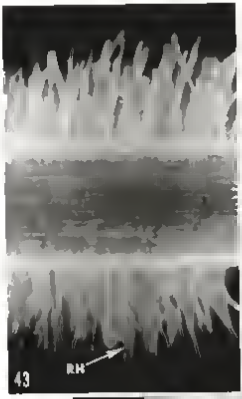

39
40
PFS
41

Fig 43 Axenically grown clover with undeformed root hairs (RH). (X 120)

Fig 44 Short, markedly deformed root hairs (RH) on clover inoculated with *R. trifolii* NA30 harvested from YEM broth at 72 h. The bacteria are flocculated in the rhizosphere and are indicated by "floc". (X 120)

Fig 45 Long, moderately deformed root hairs on clover inoculated with *R. trifolii* NA30 harvested from YEM broth at 72 h. The bacteria are flocculated in the rhizosphere and are indicated by 'floc'. (X 120)



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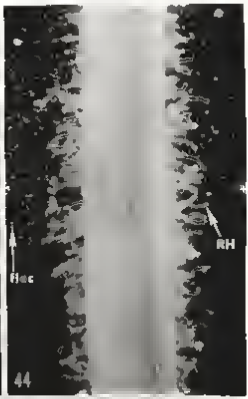

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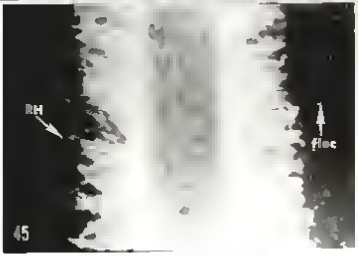


45

variety Saltzine (berseem or called clover). Fig. 44 shows deformed root hairs on clover inoculated with *R. trifolii* NA30. The bacteria flocculated in the rhizosphere and appeared as large clumps. The intensity or degree of root hair deformation could be correlated with the number of rhizobia inoculated into the rhizosphere. When the inoculum contained greater than 10^6 flora/ml, the root hairs were markedly deformed and crumted to approximately one half the length of uninoculated root hairs (as seen in Fig. 44). These crumted root hairs were less than 0.3 mm long. With lower cell counts (less than 10^5 floca/ml), root hairs were moderately curled and as long as, or longer than, uninoculated root hairs (Fig. 45). These moderately curled root hairs were greater than 0.6 mm long. Infection threads did occur in long root hairs, but high infection thread counts (greater than 10 infection threads per microscope field with the 10X objective) were restricted to areas along the root where root hair growth was stunted.

The characteristic curled root hair tip, which is referred to as a shepherd's crook, is seen in Fig. 46. Most infection threads originated in the shepherd's crook, as seen in Fig. 47. When inocula were prepared from cells cultured in clover root exudate, the incidence of infection thread initiation in undeformed root hairs was approximately 10%. When the infected root hair did not have a shepherd's crook, a light refractile bacterial flat could be seen at the infection thread origin.

Newly initiated infection threads contracted only slightly with

Fig 46 Tightly curled root hair tips (shepherd's crooks, SC) on clover inoculated with *R. trifolii* NA30 harvested from YEM broth at 72 h. (X 1,650)

Fig 47. Infection thread (IT) in a root hair with a shepherd's crook (SC) formation. The seedling was inoculated with *R. trifolii* NA30 harvested from YEM broth at 72 h (X 1,650)

46

47

the cytoplasm of the root hair (Fig. 48). As the infection thread aged with time, it became more light refractile and distinct. The root hair nucleus could be observed at the tip of the growing infection thread (Fig. 49).

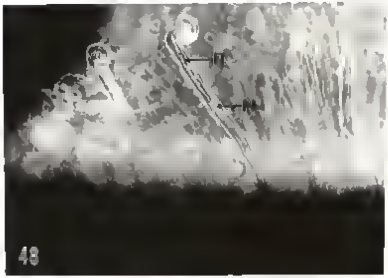
Rhizobium trifolii NA30 grown in association with clover could be found either encapsulated or unencapsulated. Fig. 50-55 show *R. trifolii* NA30 in the clover rhizosphere with capsules of various sizes. As seen in Fig. 50 and 55, a capsule could contain more than one bacterial cell. The inclusion of several bacteria within a single capsule was observed only when *R. trifolii* NA30 was grown in association with clover or clover root exudate (Fig. 30-42). Serial sections of these capsules which contained more than one cell showed that the fibrillar material completely surrounded the bacteria. Bacteria could be observed embedded in an amorphous material attached to the root hair surface as seen in Fig. 54. Fig. 55 shows capsules which appear to be two separate yet attached capsules. *Rhizobium trifolii* NA30 cells in the rhizosphere were pleomorphic only when enclosed in slime, otherwise the cells had a fairly uniform rod morphology.

Rhizobium trifolii NA30 was observed attached to root hairs in a polar orientation as seen in Fig. 56. As pointed out in the Literature Review (see page 10), the molecular basis for polar attachment is unknown. When the chemical identity of receptor sites for bacterial attachment is known, specific cytochemical tests can be used in studying attachment with the electron microscope. The thin section seen in Fig. 57 shows fibrils radiating from the cell. This material stained positive for polysaccharide with the periodic acid-silver hexamine stain (64).

The light microscope has been invaluable in studying such initial steps in the symbiosis as root hair curling and bacterial adsorption.

Fig. 48. Root hair containing an infection thread (IT) which is being led toward toward the root cortex by the root hair nucleus (N). (X 450)

Fig 49 Higher magnification of Fig 48 showing the infection thread tip (IT) and the root hair nucleus (X 1,850)


IT
48


IT
NUCLEUS
49

Fig. 50 A thin section of encapsulated bacteria within the clover rhizosphere. This section shows an encapsulated bacterium and a capsule which contained two bacteria (X 22,750)

Fig. 51. A thin section of an encapsulated bacterium in the clover rhizosphere (X 20,000)

Fig. 52 A thin section of unencapsulated and partially encapsulated bacteria in the clover rhizosphere. (X 14,560)

Fig. 53 A thin section of unencapsulated and encapsulated bacteria in the clover rhizosphere outside a root hair. (X 10,920)

Fig. 54. A thin section of an encapsulated bacterium embedded in an amorphous material attached to the root hair cell wall (X 10,920)

Fig. 55 A thin section of capsules which contained more than one bacterium. One capsule has a segmented appearance. (X 14,560)

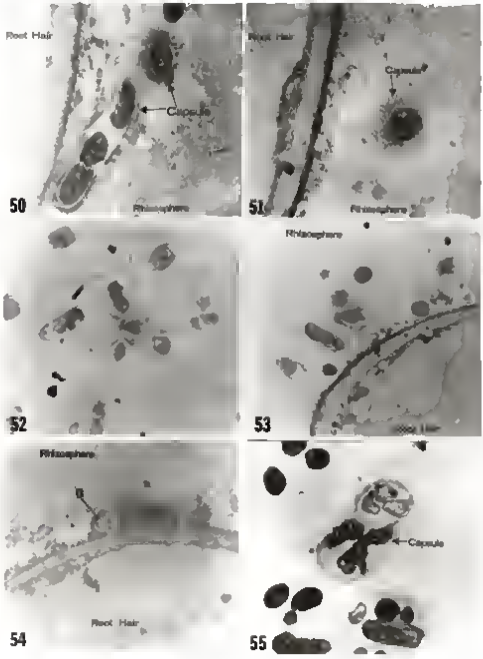

Root Hair
Capsule
Rhizosphere
50
Root Hair
Capsule
Rhizosphere
51
Rhizosphere
52
53
Rhizosphere
Root Hair
54
Capsule
55

Fig. 56. A root hair with *R. trifolii* NA30 attached in a polar orientation. The arrow indicates an attached bacterium. (X 1,350)

Fig. 57. An electron micrograph of a thin section of a polarly attached *R. trifolii* NA30 cell. (X 84,000)


56

57

In addition, the light microscope has been used to study the growth of the infection thread through the root hair. However, the point of entry, and thus the physical mechanism, cannot be resolved with the light microscope. In this study, seedlings were prepared for electron microscopy in order to section root hairs and determine if there was an invagination. The technique of serial sectioning was used so the root hair could be examined in it entirety

Root hairs having the shepherd's crook at the infection thread origin were serially sectioned, and in every case, the root hair cell wall was invaginated. There were no breaks in the root hair cell wall at the point of entry, and the root hair cell wall was continuous with the wall of the infection thread

Fig 58 is a diagrammatic illustration of a sectioned root hair based on a serial section sequence from which Fig. 59-61 were selected. The invagination was seen before, through, and past the pore (Fig 59, 60, and 61, respectively) Bacteria were seen within the pore and in the infection thread This infection thread may have been newly initiated before the fixative, as it had not progressed far into the root hair, and the nucleus as seen in close association with the tip of the infection thread

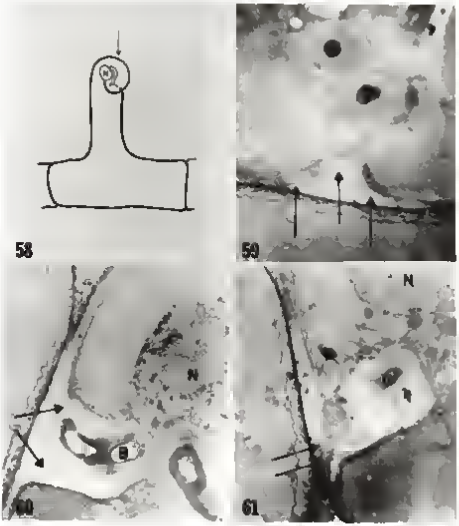
As shown in this serial section sequence, the infection thread wall at the point of invagination is difficult to see (indicated by arrows in Fig. 59-61). However, the infection thread walls away from the point of invagination are clearly recognizable This may reflect a physical and/or chemical alteration of the cell wall structure at the invagination origin, where the specific bacteria-plant interactions resulted in the initiation of an infection thread.

Fig 58 A diagrammatic illustration of a serial sectioned root hair showing the infection thread (IT), nucleus (N), and the initiation of nucleoling (top arrow)

Fig 59 A serial thin section before the invagination showing the infection thread which contained bacteria (B) The arrows indicate the region of the root hair wall where the invagination process has begun (X 11,120)

Fig 60 A serial thin section through the middle of the invagination showing the infection thread wall (arrows) of the pore, bacteria (B) within the infection thread, and the root hair nucleus (X 11,120)

Fig 61 A serial thin section past the pore, the arrows point out where the wall of the pore is erased by the knife (X 11,120)


58
59
N
B
60
N
61

Serial sections through a root hair with an infection thread which did not originate in the tightly curled shepherd's crook are shown in Fig. 63-69. Fig. 62 is a diagrammatic illustration of the root hair based on serial sections from which the sequence in Fig. 63-69 was selected. The root hair was slightly curled. The infection thread originated midway on the root hair and branched during growth through the root hair. Each branch grew into the base of the root hair. The root hair nucleus was positioned next to the branch point at the time of fixation. A floc of bacteria was attached to the root hair at the origin of the infection thread. The sectioning began at the floc (arrow) and continued through the root hair. The slime wall of the attached floc was grazed (Fig. 63), and then bacteria were revealed inside (Fig. 64). Several sections were cut through the floc before the root hair wall was sectioned (Fig. 65). The arrows in Fig. 65 indicate the interface between the root hair wall and the floc. The floc had a segmented appearance (Fig. 65 and 66). As sectioning continued progressively through the root hair, an invagination of the root hair wall became apparent (Fig. 66). Sectioning through the area where the infection thread originated revealed a pore filled with and surrounded by the floc (Fig. 67). The wall of the root hair was continuous with the infection thread wall (Fig. 67-69). The floc decreased in size past the middle of the pore (Fig. 68) and ended as the back wall of the pore was grazed by the knife (Fig. 69). Bacteria were seen within the infection thread (Fig. 67-69).

The nucleus of the root hair (Fig. 70) was positioned by the infection thread (from Fig. 63-69). The nucleus is thought to have preceded the infection thread down to the next cell layer in the root cortex, and then to have migrated to this point before fixation occurred.

Fig. 62. A diagrammatic illustration of a serial sectioned root hair with an infection thread which did not originate in a shepherd's crook. The infection thread (IT) originated midway on the root hair and branched below the nucleus (N). A floc of bacteria (F) was attached to the root hair at the origin of the infection thread. Sectioning began at the floc (indicated by the top arrow) and continued through the root hair.

Fig. 63. The slime wall (arrow) of the attached floc is grazed by the knife in this serial thin section. Unencapsulated *R. trifolii* NA30 are seen in the rhizosphere. (X 9,270)

Fig. 64. A serial thin section in which the floc is sectioned and the bacteria are revealed inside. The arrow indicates the slime surrounding the floc. (X 9,270)

Fig. 65. A serial thin section in which the root hair wall is grazed by the knife. The arrows indicate the interface between the root hair and the attached floc. (X 9,270)

62

63

64

65

Fig. 66. A serial thin section showing where the root hair wall is beginning to invaginate (arrows). The attached floc has a segmented appearance. (X 11,500)

Fig. 67. The root hair wall is invaginated to form the infection thread in this serial thin section. The plant cell wall (arrows) is continuous with the wall of the infection thread. Bacteria are seen within the floc and the infection thread. (X 8,000)

Fig. 68. A serial thin section past the middle of the invagination. The plant cell wall is indicated by the arrows. (X 11,500)

Fig. 69. A serial thin section in which the back wall (arrows) of the invagination is grazed by the knife, the attached floc has ended at this point. (X 12,400)

66

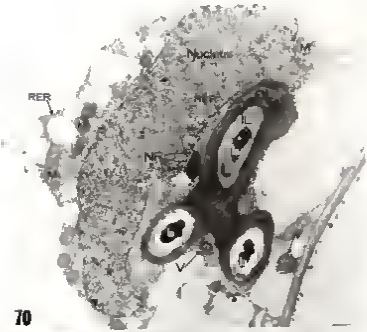
67

68

69

Fig 70 A thin section of the root hair nucleus which is positioned next to the infection thread The infection thread is composed of two distinct layers, an outer fibrillar layer (OL) and an inner amorphous layer (IL) Bacteria (B) are seen within the infection thread The nucleus had numerous nuclear pores (NP) The cytoplasm surrounding the infection thread contained mitochondria (M) and rough endoplasmic reticulum (RER) Vesicles (V) were seen fusing with the root hair cell wall and the infection thread. (X 10,200)

Fig 71 A thin section showing the branch point (indicated by arrows) of the infection thread (IT) One branch of the infection thread crossed from the root hair cell into the next cell layer in the root cortex. There is no disruption in the continuity of the infection thread walls or the plant plasmalemma (X 10,200)



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This would be in accordance with observations by Fahraeus (25) and Nutman (60) that the root hair nucleus precedes the growing infection thread into the base of the root hair cell. Fig. 70 shows numerous nuclear pores in the nuclear envelope. Vesicles containing globular inclusions were seen fusing with the root hair cell and with the infection thread wall.

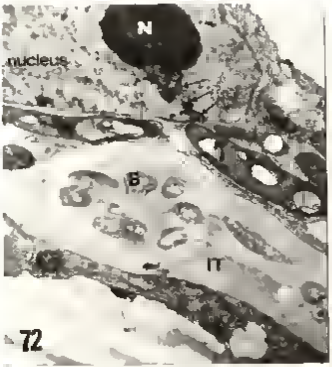
The infection thread, as seen in Fig. 70, was composed of two distinct layers, an outer layer and an inner amorphous layer, surrounding the bacteria. The inner amorphous layer was unstable in the electron beam and would be missing in thinner sections. The outer layer of the infection thread and the plant cell wall appeared similar in appearance. Both walls stained positive for polysaccharide with the periodic acid-silver hexamine stain (54). However, the inner layer did not stain. If this layer contained polysaccharide, it was not sensitive to periodic acid oxidation.

Fig. 71 shows the branch point of the infection thread (indicated by arrows) with one branch crossing into the underlying cell. The process of the infection thread crossing from one cell into another in the cortex appeared to be a repetition of the invagination process which occurred at the original site of infection. There was no disruption in the continuity of the infection thread, and the wall of the infection thread and the plasmalemma were continuous.

Fig. 72 and 73 show infection threads adjacent to nuclei; in each instance there was an accumulation of darkly staining, diffuse material between the nucleolus and the nuclear envelope. This may represent transfer of ribonucleoprotein from the nucleus to the cytoplasm. It has been thought by several workers (25,60) that there is a direct

Fig. 72 A thin section of an infection thread (IT) adjacent to the plant cell nucleus. The nucleus contained a prominent nucleolus (N) which had a diffuse, darkly staining material associated with it (unlabeled arrows). The bacteria within the infection thread are indicated by B. (X 12,600)

Fig. 73 A thin section of an infection thread (IT) which grew into the base of the root hair. The root hair nucleus was adjacent to the root hair tip. The nucleolus (N) had a diffuse, darkly staining material associated with it (unlabeled arrows). The bacteria within the infection thread are indicated by B. (X 12,600)


N
nucleus
B
IT
72


IT
N
B
NUCLEUS

biochemical communication between the plant cell nucleus and the growing infection thread as the nucleus precedes the growing tip of the infection thread into the base of the root hair cell

Fig 72 and 73 were taken from separate serial section sequences. In each case the nucleolus was a prominent nuclear structure Fig 73 was taken from the serial section sequence after the larger part of the nucleolus was sectioned In this case the nucleolus appeared smaller in this section

DISCUSSION

A study of the ultrastructure of *R. trifolii* NA30 was undertaken to determine if the organism passes sequentially through distinct morphological forms that could constitute a life cycle as proposed by several early *Rhizobium* workers.

Rhizobium trifolii NA30 had a mean generation time of 3 h when cultured in YEM broth. The rods were the most uniform in size and shape during mid-exponential growth in contrast to cells grown in soil extract and root exudate, the cells were exclusively rod shaped, no cocci were detected using the electron microscope. As the cells entered stationary phase, there was a tendency for the bacteria to become recoid. Asymmetrical cell division during growth resulted in rods of various lengths. Binary fission was the only mode of cell division observed.

In soil extract *R. trifolii* NA30 had a mean generation time of 3.5 h. The concentration of soil extract did not affect the generation time of the bacteria, but did effect the total cell yield. *Rhizobium trifolii* NA30 was not observed to go through any complex life cycle when cultured in soil extract. Binary fission was the only mode of cell division observed. The uniform rods of the inoculum harvested from YEM broth became rods of various lengths through asymmetrical divisions. Coryneforms were produced during growth in soil extract. These small cells were motile at the poles of the rods at 6 h during exponential growth but were not detected free in the medium until 36 h.

One unifying feature of the life cycle proposed by various early workers was that the coccus or swarmer was the infective form of *Rhizobium*. Table 5 shows that after 10 h, inoculs prepared from soil extract began to decrease in infectivity. Cells were not detected free in the medium until after 36 h, at which time the infectivity was significantly reduced.

In root exudate *R. trifolii* NA30 had a mean generation time of 4 h. Seed root exudate in the medium caused a decrease in optical density and dry weight measurements as the cells entered the stationary phase. There was no decrease in optical density with cells cultured in root exudate treated with PVP to remove the seed root exudate. The neutralization of root exudate did not affect the growth rate of the bacteria but did affect the total cell yield. The addition of clover seed extract to root exudate caused a decrease in the generation time. The reason for this decrease in generation time is unknown.

As with YEM broth and soil extract, no complex life cycle was observed in root exudate and binary fission was the only mode of cell division observed. Cells cultured in root exudate did produce corni, but these small cells constituted less than 5% of the total population of cells. Cells cultured in root exudate became heavily encapsulated and were associated with slime. This was unique to cells cultured in root exudate and was not seen when the cells were cultured in YEM broth or soil extract.

The rhizobia were pleomorphic and were subject to aberrant cell divisions. However, the pleomorphic forms did not constitute a life cycle but rather were a response to growth conditions such as nutrient limitations. In YEM broth, soil extract, and root exudate the bacteria were predominantly bacillary. Under the light microscope banded rods

rould br seen in all three media The banded appearance was attributed to the accumulation of PHB and the darkly staining polar bodies It is believed that these banded rods correspond to the "gonidangia" observed by early workers, and the polar bodies correspond to the previously described "gonidia. if a cell lysed and released the polar bodies into the medium, it would appear, under the light microscope, as if a "mother cell' had released 'swarmers." It is reminded that the life cycle of the rhizobia, as proposed by some early *Rhizobium* workers, was an erroneous interpretation of stains prepared for the light microscope

infectivity studies showed that the morphology of the inoculum could not be correlated with infectivity Inoculum prepared from stationary phase cells in YEM broth was significantly more infective than was inoculum prepared from exponentially growing cells in the same medium. Root hair curling ability and bacterial adsorption did not account for the differences in infectivity

According to the "polygalacturonase" theory of Ljunggren and Fahraeus (48), the extracellular polysaccharide of infective rhizobia plays an important role in specificity and infection With *R. trifolii* NA30, stationary phase cells produced more extracellular polysaccharide than did exponentially growing cells However, in preparing the inoculum, cells were centrifuged from YEM broth and resuspended in sterile PBS For this extracellular polysaccharide to influence infection, it would have to be active at low concentrations This is not unreasonable as plant hormones are active at concentrations as low as 10^{-12} M for Indole-3-acetic acid (39) it is unknown at this time, what specific function, if any, the extracellular polysaccharide has in the infection process

The seedling was inoculated with bacteria when the Fahraeus glass slide assemblies were prepared. Stationary phase cells may have survived this initial starvation period, before plant root exudates accumulated, better than an inoculum prepared from cells harvested at mid-exponential growth from YEM broth. As indicated in Table 3, the number of bacteria in the inoculum did affect the mean number of infection threads formed. The difference in the number of infection threads formed from stationary phase cells and exponentially growing cells would indicate that the population of exponentially growing cells would have to be reduced by an order of magnitude of three to account for the difference in infectivity.

Cells cultured in soil extract did not change their infectivity while growing exponentially, however cells in stationary phase produced fewer infection threads per seedling. Inspection of the rhizosphere of seedlings inoculated with stationary phase cells revealed no obvious reasons why these cells would be less infective than exponentially growing cells.

The opposite effect was seen for cells cultured in root exudate. Inocula prepared from stationary phase cells produced significantly more infection threads than did inocula prepared from exponentially growing cells. This enhancement of infectivity may have been due to either the growth phase of the cells or the increased time of exposure of the bacteria to root exudate. These two possibilities could not be differentiated during the experiment. Light microscopic examination of the rhizosphere of seedlings inoculated with root exudate cultured cells showed an increased number of bacterial films attached to root hairs. The incidence of infection thread initiation in undeformed root

hairs was doubled over that seen [illegible] Inoculum prepared from stationary phase *R. trifolii* NA30 cultured in root exudate had more attached bacterial flacs and infection threads in undeformed root hairs than did seedlings inoculated with exponentially growing cells from root exudate.

Infectivity of an inoculum could not be correlated with the morphology of the cells. The swarmer form of the rhizobia was proposed as a means of explaining how the bacterial cell penetrated the plant cell wall. Beijerinck (3) determined that *Rhizobium* did not hydrolyze cellulose. The question arose as to how bacteria which could not degrade the plant cell wall could infect the plant cell. McCoy (55) confirmed that the rhizobia did not hydrolyze cellulose or pectin when cultured in media containing these substrates.

The large scale production of hydrolytic enzymes would be detrimental to the establishment of the symbiosis as the rhizobia would have the potential to be pathogenic. The infection of the legume by the rhizobia must proceed in such a manner that the physiology of the plant is affected as little as possible. A major disruption of the host cell physiology results in abortion of the infection process (60).

Ultrastructural evidence presented by this research shows that the bacteria enter the root hair by a process of invagination. The bacteria redirect the growth of the plant cell wall so that the root hair grows back into itself. In this way, the bacteria enter the root hair through a bacteria-induced, plant synthesized infection thread which remains from the invagination process. The bacteria remain extracellular while inside the root hair.

Infection threads on seedlings inoculated for seven days were

examined and the pores were open. At this time it is unknown if the pores remain open for a longer period of time. The pore was not directly exposed to the rhizosphere but was either encircled by the shepherd's crook (Fig. 59-61) or plugged with a floc of bacteria (Fig. 63-65); in this way, entry into the infection thread would be limited to those bacteria trapped in the shepherd's crook or to the bacteria comprising the floc. This may explain the infrequent occurrence of isolation of more than one strain of *Rhizobium* from one nodule.

The majority of infected root hairs have the tightly curled root hair tip, but infection will occasionally occur in a relatively undeformed root hair. It is believed that the attached bacteria(i) (Fig. 62) served the same function as the tightly coiled root hair tip (Fig. 58), localizing and concentrating the biochemical interactions of the plant and bacteria to a threshold level which is required for initiation of infection.

Seedlings inoculated with root exudate cultured bacteria showed an increased number of infection threads originating from undeformed root hairs. These infection threads originated from attached bacterial flocs. It is proposed that culturing the bacteria in clover root exudate increased the encapsulation of the bacteria which enabled the bacteria to infect a greater number of root hairs. In addition, these encapsulated bacteria may have stimulation infection to occur in less time than cells cultured in YEM or cell extract.

Future studies on infection should concentrate on determining what role the extracellular and capsular polysaccharides play in the infection process and the specific biochemical interactions which allow infection to take place and result in the invagination of the plant cell wall.

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BIOGRAPHICAL SKETCH

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She has one child, Anne-Marie Ellen Napoli.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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Associate Professor of Soil Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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